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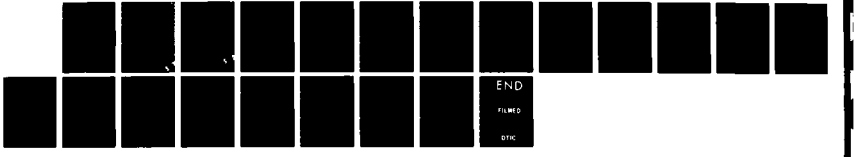

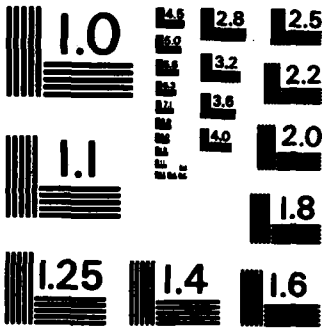

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This research program is concerned with the long-term consequences of prolonged elevation of acetylcholine on cholinergic and non-cholinergic transmission in hippocampal synapses and the mechanisms through which any such effects might be achieved. Progress has been made made in three areas: (1) a cholinergically mediated physiological response has been identified in the in vitro hippocampal slice, (2) the response of the hippocampus to repeated applications of cholinergic agonists has been found to be relatively constant, particularly when compared to that elicited by activation of two types of receptors for acidic amino acids, and (3) the stimulation of a potentially very potent second messenger system (turnover of phosphatidylinositol) by cholinergic agonists was discovered to be completely blocked by concurrent activation receptors for amino acid transmitters. These results point to the conclusions that the cholinergic receptor is not particular labile and that its interaction with its second messenger target system is tightly regulated by noncholinergic inputs. This raises the possibility that the long-term effects reported in the literature for cholinergic stimulation require prolonged exposures to the transmitter and/or are dependent upon the status of non-cholinergic inputs.

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EFFECTS OF ACETYLCHOLINESTERASE INHIBITION ON CHOLINERGIC TRANSMISSION IN THE IN VITRO HIPPOCAMPAL SLICE

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INTERIM SCIENTIFIC REPORT
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G. Lynch

1. Summary:

Three groups of experiments were conducted in an effort to identify the mechanisms through which acetylcholine exerts long-term effects on brain cells.

(a) A cholinergic pathway was identified and studied physiologically using the in vitro hippocampal slice technique. This connection consists of a group of axons that activate interneurons, which in turn inhibit the activity of one of the two primary classes of hippocampal neurons. The system provides a simple model with which to study cholinergic transmission in brain.

(b) The relative "stability" of the brain cholinergic receptor was tested by treating slices of hippocampus with a series of brief pulses of cholinergic agonists and measuring several physiological parameters. These results were compared with those obtained with a series of exposures to excitatory amino acids, including several thought to be neurotransmitters. Cholinergic compounds produced depolarization and an increase in membrane resistance; their effects were quite constant across several infusions. Some, but not all, noncholinergic agonists desensitized after two or three applications. These results, combined with pharmacological data, led to a new four part classification scheme for amino acid receptors in brain.

(c) Cholinergic agonists applied to brain slices triggered a massive increase in the rate of phosphatidylinositol (a membrane phospholipid) turnover. This biochemical reaction is thought to mediate trophic reactions in a number of cell types. Activation of a second, non-cholinergic receptor, was discovered to completely block the effects of cholinergic drugs on phosphatidylinositol turnover, indicating that a novel mechanism exists for regulating the actions of acetylcholine.

Experiments currently in progress will (1) identify concentrations of cholinergic agonists that produce long-lasting effects using the repeated application paradigm,

and (2) determine if the increased PI turnover produced by acetylcholine stimulates the enzymes protein kinase C and calpain. This will be followed by experiments to test the hypothesis that the second-messenger biochemistry activated by cholinergic agonists is responsible for lasting structural and physiological changes in cholinergic and non-cholinergic synapses in brain.

2. Goals

Three specific aims, each at a different level of analysis, were followed: (1) identify physiological responses elicited by electrical stimulation of cholinergic projections in brain, (2) measure the physiological effects of repetitive applications of cholinergic agonists on neurons and compare with those elicited by other putative transmitter substances, and (3) investigate novel "second messenger" chemistries activated by cholinergic-agonists and attempt to link these with processes that might produce pathology.

3. Status of the Project

There is considerable evidence that prolonged exposure of various types of cells to acetylcholine or its analogues produces effects that in some cases can be described as trophic and in others as pathological. An obvious example is provided by the neuromuscular junction where application of cholinergic agonists for more than a few minutes results in the atrophy and degeneration of muscle fibers (Salpeter, 1982). Atrophy and degeneration have also been recorded in brain after prolonged exposure to acetylcholine (Olnen et al, 1983). Thus, while acetylcholine serves a transmitter role, it also has considerable potential for creating long-lasting and widespread cellular reorganization. This might be used as part of the brain's adaptive responses to changing circumstances; it is also possible that the trophic-like effects of acetylcholine are responsible for lasting disturbances in brain functioning found in some diseases thought to involve acetylcholine (e.g. Alzheimer's) or after exposure to agents that perturb

cholinergic transmission (e.g. organophosphorus inhibitors of acetylcholinesterase).

This research program has been concerned with identifying conditions (concentrations, exposure time, single vs repeated applications) under which cholinergic receptor stimulation produces long-term effects on the anatomy and physiology of brain cells and to identify the biochemical mechanisms responsible for such effects.

(a) Identifying cholinergically mediated responses

With few exceptions, cholinergic projections in the mammalian brain are diffuse (Lynch et al, 1977, for a review) and exceedingly difficult subjects for physiological studies. It is not surprising then that so little is known about the properties of cholinergic transmission in brain. The septo-hippocampal projections form the best-defined (anatomically and biochemically) cholinergic system in mammalian forebrain and one branch of this projection (that to the dentate gyrus subdivision of hippocampus) is relatively dense and forms a laminated synaptic field.

These cholinergic septal fibers terminate in a layer (the infragranular layer or "IGL") filled with interneurons, some of which form "basket" endings with the dentate gyrus granule cells (Lorente de No, 1934). There is ample evidence that these types of contacts are inhibitory in function (Andersen et al, 1964). From this anatomy we would expect activation of the cholinergic fibers to indirectly alter inhibitory influences in the granule cells by producing synaptic effects on local inter-neurons.

To test this, in vitro slices were prepared with one stimulating electrode placed in the perforant path, that fiber projection which provides the major excitatory input to the granule cells, and a second along the trajectory of the cholinergic septal fibers. A recording electrode was then lowered into the granule cells to monitor extracellular responses. The field potential generated

by activation of the perforant path consists of a large positive response broken by a sharp negative potential (see Deadwyler et al, 1975); the first of these is due to the dipole generated between the cell bodies and summed excitatory post-synaptic potentials in the dendrites while the latter reflects the extracellular currents generated by the near-simultaneous all-or-none discharges (spikes) of a large number of granule cells ("the population spike"). Stimulation of the septal projections to the dentate gyrus produced a profound inhibition of the population spike elicited by perforant path actuation without detectably changing the positive response (Fig. 1). This effect was found when the IGL stimulation preceeded the perforant path activation by as little as 2.0 msec indicating that not more than two synapses were involved. Stimulation at a variety of positions above and below the IGL was ineffective.

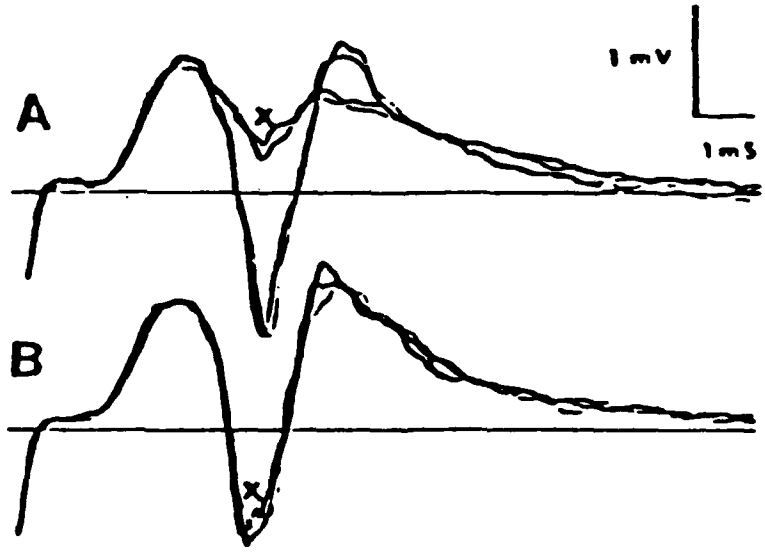

Figure 1. (A) Shown are computerized reconstructions of extracellular responses of the dentate gyrus granule cells to stimulation of the perforant path alone or stimulation of the perforant path preceded (10 msec) by activation of fibers passing through the infragranular layer. The population spike (the summed discharges of several granule cells) is the prominent negative-going wave that bisects the slower positive wave (this positive wave is the extracellular correlate of the EPSP's generated in the dendrites of the granule cells by the perforant path stimulation). The population spike was selectively depressed on the trials ("x") in which IGL stimulation was used. (B) Effects of atropine sulfate (30 μM) on the perforant path response and the inhibition of the population spike produced by IGL stimulation. As shown, IGL stimulation ("X") had virtually no effect on the perforant path response including the population spike in the presence of atropine.

Having obtained a reliable effect we tested for the involvement of acetylcholine by infusing atropine through the slices. As shown in figure 2, atropine totally blocked the inhibitory response of infragranular stimulation on the population spike. Atropine's effects were completely reversible, as shown in figure 2. Note also that the drug did not alter the response of the granule cells to perforant path activation but only the inhibitory effects of preceding infragranular stimulation on that response. Finally atropine acts in a dose-dependent fashion with a threshold concentration between 5 and 10 μM (figure 3). Scopolamine works in the same fashion as atropine but requires higher concentrations.

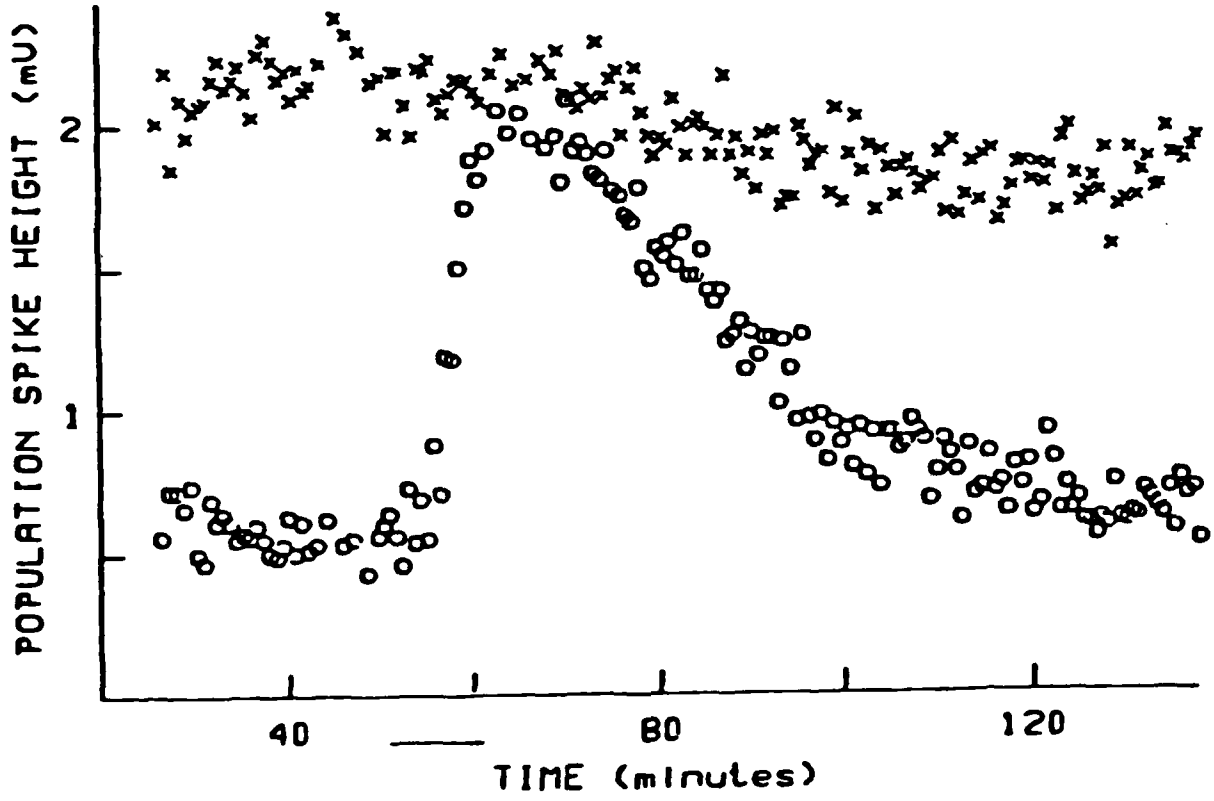

Figure 2. A typical experiment measuring the effects of IGL stimulation on the perforant path evoked potential in the presence and absence of atropine. The perforant path was stimulated once every 20 seconds with (O's) and without (X's) preceding IGL stimulation. Note that the IGL activation greatly reduced the amplitude of the spike. The thin horizontal line below the time axis denotes the period during which atropine was perfused through the slice. As is evident, the drug had no effect on the response to perforant path stimulation alone but virtually eliminated the powerful inhibitory effect of IGL stimulation. With time, atropine washes out of the slice and the IGL inhibition returns.

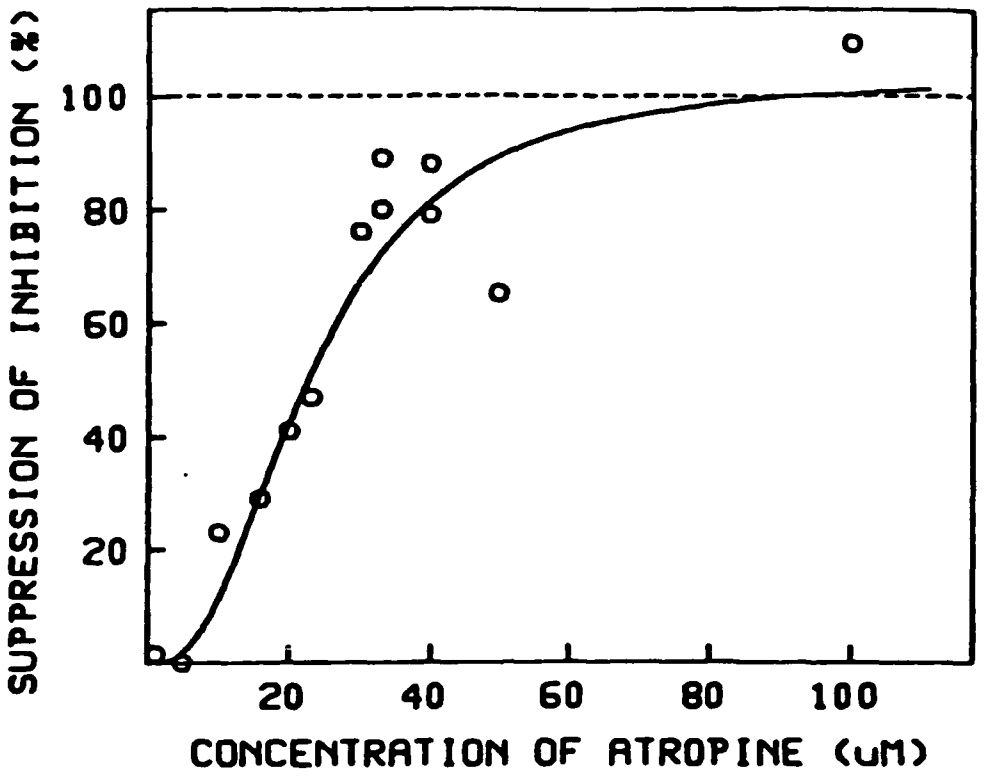

Figure 3. Dose response curve for the effects of atropine on the population spike inhibition produced by IGL stimulation.

The above results indicate that activation of the cholinergic axons in the IGL suppresses the discharge of dentate gyrus granule cells. To better characterize this effect we used intracellular recording techniques. IGL stimulation produced a sizeable hyperpolarization the amplitude of which was dependent upon the membrane potential. This hyperpolarization blocked the spike discharge evoked by perforant path stimulation. Electrodes filled with potassium acetate (KAc) or potassium

chloride are known to block inhibitory post-synaptic potentials. Apparently enough of the filling solution escapes through the tip of the electrode to alter the reversal potential for chloride, the ion species that carries the hyperpolarizing current (Eccles et al., 1976). We have found that the hyperpolarizing potential produced by IGL stimulation is reversed by KAc electrodes. While further studies are needed, we conclude that IGL stimulation elicits classical inhibitory postsynaptic potentials (IPSP's) in the granule cells.

Many, perhaps most, inhibitory interneurons in brain use GABA as a transmitter and there is strong evidence that this is true for the basket cells of the dentate gyrus (Fonnum, 1976; Nadler et al., 1974a). If stimulation of septal-cholinergic axons stimulates an intermediary cell that releases GABA, drugs that block GABA (and thereby causes an IPSP) receptors should block the effects of IGL stimulation. We tested this prediction by infusing the slices with picrotoxin, a potent and selective antagonist of one of the two classes of GABA receptors. This drug totally eliminated the suppression of granule cell discharge produced by IGL stimulation.

Recently, Cole and Nicoll (1983) reported on the physiology of a second component of the septo-hippocampal system in slices; namely, that which terminates in the pyramidal cell field CA1. Thus two cholinergic pathways have now been identified in the hippocampus in vitro that are acccessible for physiological, pharmacological, and neuroanatomical investigations.

(2) Physiological responses elicited by repeated applications of cholinergic agonists and acidic amino acids.

These experiments tested if repetitive applications of cholinergic and non-cholinergic agonists produce rapidly appearing but long-lasting changes in the physiology of hippocampal neurons. In addition to providing an answer to

this question, the work funded by AFOSR led to the discovery of a classification scheme for the acidic amino acid receptors in hippocampus and provided important information as to which receptor is actually used in synaptic transmission by the major intra-hippocampal circuitry,.

The first experiments used extracellular electrodes to record synaptic and antidromic responses in the field CA1 of the in vitro hippocampus in the presence and absence of drugs added to the perfusion lines. A variety of acidic amino acids, some of which have been suggested to be neurotransmitters, were first tested (glutamate, aspartate, cysteine sulfinic acid (CSA), quinolate, n-methyl-d-aspartate (NMDA), homocysteate, and kainate). All of these reversibly depolarized the target cells as evidenced by reductions in the antidromic responses and the virtual disappearance of the synaptic responses. Upon restoration of perfusion with control medium the responses quickly returned to normal. Successive applications resulted in the appearance of a desensitization to all of the amino acids except homocysteate and kainate; that is, the slices no longer responded at all to previously effective dosages of glutamate, etc. This desensitization was itself reversible and responses to the amino acids reappeared by about 1 hour after the last of a series of closely spaced applications. It is important to note that in no case was desensitization accompanied by any change in the synaptic responses elicited by stimulation of hippocampal fibers. Thus, when the slices were totally unresponsive to glutamate, etc., the potentials produced by activation of synapses were essentially normal. This strongly suggests that the desensitized receptors were different from the transmitter receptors.

These results divided amino acid receptors in hippocampus into two groups; desensitizing and non-desensitizing. By combining these categories with those reached on the basis of pharmacological experiments, we arrived at four classes of receptors; the characteristics of one of these, that which binds homocysteate,

closely resemble those of the receptor for the endogenous synaptic transmitter.

These conclusions were re-examined using a completely different experimental paradigm involving the biochemical measurement of sodium fluxes in slices of hippocampus. All of the amino acids which cause physiological excitation also produce an increase in sodium flux across membranes; however, the responses elicited by several were substantially reduced by lowering the calcium concentration in the medium or by removing large numbers of axon terminals, suggesting that the amino acids in this group operate presynaptically to release neurotransmitter. Homocysteate's actions were unaffected by removal of calcium or denervation, indicating again that this amino acid operates on a post-synaptic site. Together with the earlier results, these findings strongly suggest that homocysteate acts on an endogenous post-synaptic transmitter receptor and that this receptor does not desensitize.

Intracellular recording was then used to compare desensitizing and non-desensitizing amino acids with the changes produced by comparable treatment with carbachol, a cholinergic agonist. We replicated the earlier pattern of results for the acidic amino acids and demonstrated that desensitization occurred without any changes in resting membrane potential, membrane impedance, excitatory post-synaptic potentials (EPSP's), or inhibitory post-synaptic potentials (IPSP's. This provides strong evidence that the effect is due to a classical desensitization of an extra synaptic receptor (Fig. 4). Carbachol (1 min. perfusion) produced a depolarization of the cells that was accompanied by an <u>increase</u> in membrane resistance; this pattern has been found for cholinergic agonists in several systems and is thought to reflect the closing of a potassium channel linked to a muscarinic receptor (the "M" channel). Interestingly, Cole and Nicoll (1983) argue that this is also the primary post-synaptic response elicited by stimulation of the septohippocampal fibers.

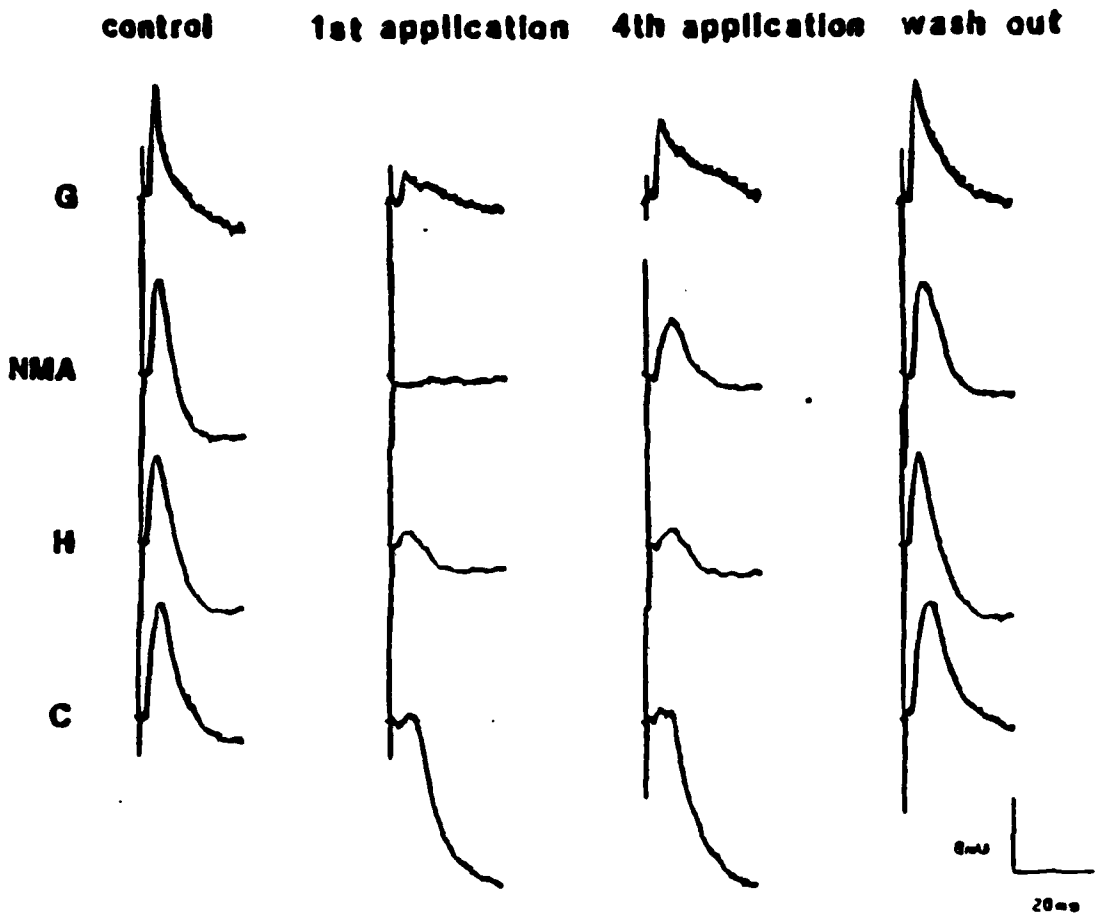

Figure 4. Intracellular recording data from four cells in hippocampal slices are shown. The upward deflections of the records are excitatory post-synaptic potentials (EPSP's) elicited by stimulation of the Schaeffer-commissural afferents of these CA1 pyramidal cells. The panels to the left are records collected before perfusing drugs into the slices. First and fourth applications refer to the first and last of a series of one minute applications (separated by 10- 15 minutes) of the following: glutamate (G), n-methyl-aspartate (NMA), homocysteate (H), or carbachol (C). Each of these compounds depolarizes the cells producing a reduction in the EPSP on 1st application; by the fourth perfusion the effects of glutamate and NMA are greatly reduced (desensitization) while those produced by homocysteate and carbachol are unchanged. Records taken 10 minutes after the last perfusion show that the EPSP's are unchanged from the control period. Thus, desensitization of glutamate and NMA receptors does not affect the receptors for the endogenous synaptic transmitter. Note that carbachol enhances the after-hyperpolarization that follows the EPSP while the acidic amino acids reduce this potential (Fagni, Baudry, and Lynch, 1984 and in preparation).

The effects of carbachol reversed quickly upon perfusion with normal medium, making it relatively easy to test repeated applications of the drug. Desensitization did not occur in any of the cells tested.

To summarize, hippocampus contains four categories of excitatory amino acid receptors and one of these has been tentatively associated with the transmitter receptor. Receptor desensitization is found for two of the categories although not for the one which includes the transmitter receptor. Carbachol blocks a potassium ion channel (the M channel) and this effect clearly does not desensitize across repetitive treatments. It must be emphasized that these results apply only to a period of about an hour after treatment and relatively modest dosages of carbochol. We are currently using longer perfusions with higher concentrations of carbachol and following physiological responses for 6-8 hours.

(3) Biochemical studies of cholinergic receptor functioning: effects on possible second messenger systems.

One of the more exciting recent developments in cholinergic pharmacology has been the discovery (see Marx, 1983, Michel, 1983) that activation of the muscarinic receptor triggers a potentially critical second messenger system involving the turnover of phaphatidylinositol (PI). PI turnover is known to be accelerated by several growth factors and may be a pathway leading to a host of trophic effects throughout the body (Bradshaw, 1984; Berridge and Irvine, 1984). The PI turnover system thus provides a means through which acetylcholine could produce long-lasting or even pathological effects in brain. To investigate this possibility, we measured PI turnover slices of hippocampus that had been exposed to physiologically effective dosages of carbachol; continuing a theme that has been followed throughout this project we compared the results with those obtained for the excitatory amino acids. Slices were incubated in 4 uCi/ml of [2-^{3}H] -inositol (15 Ci/mmol) for 45 minutes and then placed in medium containing carbachol (0.4mM) or agonists for other receptors. The incubations were stopped by placing the

slices in 1:2 (vol/vol) chloroform/methanol. The samples were homogenized by sonication and a 2 phase system obtained by adding 300 ul chloroform and 300 ul of water. The phases were separated by centrifugation and samples of the upper (aqueous) phase collected and run on an anion exchange column. These were washed with water to remove [myo^{3}H]-inositol -- labelled myo-inositol-1 phosphate was eluted with ammonium formate/formic acid and counted. This assay measures the extent to which inositol is converted into one of its major metabolic products.

As shown in Table II, carbachol produces a marked increase in inositol turnover while n-methyl-d-aspartate (NMDA), a potent agonist for one type of extra-synaptic amino acid receptor, has virtually no effect. This was as expected but we were quite surprised to find that when the two agents were combined the effects of carbachol were complete blocked (Table II). To test the pharmacological specificity of this effect, we combined carbachol and NMDA in the presence of aminophosphonovalerate (APV), a potent antagonist of the NMDA receptor. Blocking the effects of NMDA with APV resulted in a return of the carbachol elicited breakdown of inositol. Depolarization with high levels of potassium has no effects on carbachol induced changes in inositol turnover, indicating that the effect of NMDA on carbachol was not due to membrane depolarization.

Thus we conclude that activation of the extra-synaptic amino acid receptor for NMDA blocks a major biochemical effect of carbachool. The functional role of the extra-synaptic amino acid receptors has long been a mystery; these results suggest the exciting possibility that they serve to regulate the effects of cholinergic synapses. It will indeed be interesting to determine if NMDA blocks the physiological effects of carbachol.

The PI turnover system has been associated with intracellular events of great significance including activation of the rate-limiting enzyme (pyruvate dehydrogenase) in mitochondrial metabolism, the release of calcium from the endoplasmic reticulum,

Table 2. Effect of N-methyl-d-aspartate (NMDA) on carbachol-induced stimulation of phosphoinositide turnover in hippocampal slices.

TREATMENT	PERCENT CHANGE FROM CONTROL
CONTROL (no treatment)	100%
CARBACHOL (0.50 mM)	342%
NMDA (0.10 mM)	95%
NMDA + CARBACHOL	100%
NMDA + APV (0.10 mM) + CARBACHOL	336%

and the activation of the phosphorylating enzyme protein kinase C (see Berridge, 1984, for a review). It is not known if cholinergic receptor stimulation activates any or all of these, or if in brain these events are regulated by factors that offset cholinergic stimulation of the PI system. We now have a suitable assay for protein kinase C and will shortly answer the question of whether this enzyme is activated by cholinergic stimulation. If carbachol, etc., do not activate kinase C, then we would have to conclude that factors exist that are critical for regulating the second messenger effects triggered by cholinergic stimulation. If, on the other hand, we find that protein kinase C is activated, then we will begin to assess what effects this might have on cell functioning. It should be noted that this enzyme is a receptor for at least one class of tumor promoters (the phorbol esters) and accordingly is being intensively studied in a number of laboratories.

There is a second and related way in which the cholinergically-driven PI turnover could produce long-lasting changes in brain cells. As mentioned, one of the products (inositol triphosphate) of the PI system causes the release of calcium from the endoplasmic reticulum - neurons contain a calcium sensitive protease (calpain) that several laboratories (including our own) have shown to degrade proteins that cross-link the elements of the cytoskeleton with each other and with the plasma membrane (Lynch and Baudry, 1984 for a review). Studies from other groups have demonstrated that protein kinase C is a substrate for calpain (Kishimoto et al, 1983) and that when degraded the kinase becomes irreversibly activated (Tapley and Murray, 1984). Given that protein kinase C phosphorylates a number of critical targets, including the membrane pump involved in regulating intracellular pH, this action of calpain could have drastic effects on the neuron. This is of particular interest in the present context because it has been shown that much of the pathology found in muscle after prolonged exposure to cholinergic

agonists is blocked by inhibitors of calpain (see Salpeter, 1982, for a review). We will test if cholinergic stimulation results in the degradation of two of the targets of calpain: protein kinase C itself and the membrane-associated protein fodrin; techniques appropriate for this work are now in use in the laboratory.

4. Experiments in Progress

(a) Testing effects of longer exposure to high concentrations of carbachol on rapidly appearing as well as delayed physiological effects in hippocampal cells.

(b) Combining carbachol with acidic amino acids known to block carbachol's effects on PI turnover and test for physiological effects on hippocampus (e.g. does NMDA block cholinergic effects).

(c) Test if there are concentrations or exposure times for cholinergic drugs, alone or in combination with excitatory amino acids, that activate protein kinase C and the proteolytic enzyme calpain.

(d) Correlate effects of cholinergic drugs on physiology with those on second messenger chemistry.

In the near future, we will attempt to manipulate the long-term effects with drugs directed at various aspects of the second messenger system.

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Chronological List of Publications

(a) Fagni, L., Baudry, M., and Lynch, G. Classification and properties of acidic amino acid receptors in hippocampus. I. Electrophysiological studies of an apparent desensitization and interactions with drugs which block transmission. J. Neurosci., 1983, 3, 1538-1546.

(b) Baudry, M., Kramer, K., and Lynch, G. Classification and properties of acidic amino acid receptors in hippocampus. III. Supersensitivity during the post-natal period following denervation. Molecular Pharmacology, 1983, 24, 229-234.

(c) Fagni, L., Baudry, M., and Lynch, G. Intracellular studies of an apparent desensitization of excitatory amino acid receptors in hippocampal slices. Abstr. Soc. Neurosci., 1984, 10, 228.

(d) Simonson, L., Baudry, M., Siman, R., and Lynch, G. Regional distribution of calcium activated protease activity in neonatal and adult rat brain. (final submission to Brain Research, copy attached).

(e) Baudry, M. and Lynch, G. Excitatory amino acids inhibit acetylcholine-induced stimulation of phosphatidylinositol turnover in rat hippocampal slices. (to be submitted to Nature, Feb.-Mar. 1985, draft copy attached).

(f) Fagni, L., Baudry, M., and Lynch, G. Effects of repetitive applications of carbachol and excitatory amino acids on the physiology of hippocampal neurons. (to be submitted to Brain Research, March 1985).

List of Professional Personnel

M. Baudry - Associate Professor (Department of Psychobiology)

L. Fagni - Post-doctoral Fellow

R. Siman - Post-doctoral Fellow

J. Larson - Graduate Student

K. Kramer - Graduate Student

Interactions

Some of the results were discussed during invited talks by the principal investigator at several universities and professional meetings. The work on desensitization was the topic of a poster at the Neurscience Society Meeting.

Inventions, patents - None

Other Information

Two developments have influenced the timing of the research and the degree to which various components have been emphasized: (1) Cole and Nicoll (see above) reported on the cholinergic septo-hippocampal projections to field CA1 at about the same time we identified the response elicited by these connections in the dentate gyrus. With two sets of results in hand, we did not feel it was necessary to spend as much time on detailing the characteristics of the cholinergic synapses as originally planned. (2) The studies on the biochemical processes triggered by cholinergic agonists went much faster than was anticipated; moreover, a discovery of great potential significance was made (i.e. that certain amino-acids totally block acetylcholine's effects on second messenger chemistry) and it seemed only appropriate to pursue it. The anatomical aspect of the program has not received the attention originally planned, although this should be partially corrected in the coming year.

END

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